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Carboxylation of *o*-Cresol by an Anaerobic Consortium under Methanogenic Conditions

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The metabolism of *o*-cresol under methanogenic conditions by an anaerobic consortium known to carboxylate phenol to benzoate was investigated. After incubation with the consortium at 29°C for 59 days, *o*-cresol was transformed to 3-methylbenzoic acid, which was not further metabolized by the consortium. Proteose peptone in the culture medium was essential for the transformation of *o*-cresol. In addition, a transient compound detected in the culture was identified as 4-hydroxy-3-methylbenzoic acid. *o*-Cresol-6_d was transformed by the consortium to deuterated hydroxy-methylbenzoic acid and deuterated methylbenzoic acid. These results demonstrate that *o*-cresol is carboxylated in the *para* position relative to the phenolic hydroxyl group and dehydroxylated by the anaerobic consortium.

The degradation of aromatic compounds under aerobic conditions has been widely studied. In contrast, the metabolism of these compounds by anaerobic bacterial populations is less well known. However, the ability of anaerobic microorganisms to degrade aromatic compounds is important (3, 5). Horowitz et al. (7) examined the anaerobic degradation of a variety of substituted aromatic compounds and showed that some were persistent.

Numerous investigators have attempted to demonstrate the degradation of *o*-cresol under anaerobic conditions. It has been shown that *o*-cresol was persistent under methanogenic (4, 7, 14, 16), denitrifying (10), and sulfate-reducing (14) conditions. In methanogenic cultures that transformed toluene, Grbić-Galić and Vogel (6) observed the transient appearance of *o*-cresol. Based on the products identified, they made the hypothesis that *o*-cresol could be transformed to 2-methylcyclohexanol by reduction of the aromatic ring or to 2-hydroxybenzoic acid by oxidation of the methyl group. A similar methyl group oxidation was recently reported in *o*-cresol-degrading, sulfate-reducing enrichment cultures (15).

Only recently, Kaminski et al. (8) succeeded in acclimating a methanogenic consortium to *o*-cresol. Béchard et al. (2) showed that a consortium that carboxylated phenol to benzoate under methanogenic conditions could also transform *o*-cresol. Since little is known about the anaerobic biodegradation of this compound, the transformation of *o*-cresol under methanogenic conditions was investigated and some of the metabolites were identified.

MATERIALS AND METHODS

Microorganisms. The anaerobic bacterial consortium used in this study is known for its ability to degrade phenolic compounds (1) and to carboxylate phenol to benzoate (2).

Growth of the consortium. The consortium was cultivated in the minimal medium of Boyd et al. (4) containing 150 mg of *o*-cresol (Baker Chemical Co., Phillipsburg, N.J.) per liter and 0.05% (wt/vol) proteose peptone no. 3 (Difco Laboratories, Detroit, Mich.). A 10-ml inoculum of phenol-degrading culture was aseptically transferred to 70 ml of fresh medium in a 100-ml serum bottle and then incubated at 29°C. After 14 days of incubation, proteose peptone was added to the culture to a final concentration of 0.5% (wt/vol). The other conditions were as previously described (2). *o*-Cresol-free controls were also used. In some experiments, proteose peptone was omitted from the culture medium. To investigate the degradation pathway, potential metabolites of the transformation of *o*-cresol, such as 2-methylcyclohexanol, 2-hydroxybenzoic acid, and 3-methylbenzoic acid (all from Aldrich Chemical Co. Inc., Milwaukee, Wis.), were added as substrates in some cultures.

Chemical analysis. *o*-Cresol and its metabolites were extracted from culture fluids with ethyl ether after acidification with concentrated sulfuric acid. The extracts were concentrated by evaporating the solvent under a stream of nitrogen, derivatized in a mixture of *N*,*O*-bis(trimethylsilyl)-trifluoroacetamide–acetonitrile (1:4, vol/vol), and analyzed by gas chromatography (GC) with a 25-m 5% phenylmethyl-silicon capillary column (Hewlett-Packard, Arondale, Pa.) (2). GC-mass spectrometry (MS) was also performed using a similar GC column and an Ion Trap 800 (Finnigan) mass spectrometer. The initial column temperature of 70°C was increased to 120°C at a rate of 10°C/min, held for 2 min, increased to 230°C at a rate of 5°C/min, and increased to 300°C at a rate of 20°C/min. The mass spectra were recorded in the repetitive scanning mode from 70 to 400 atomic mass units. Identifications were made by comparison of the retention times and the mass spectra of the extracted products with those of authentic standards such as 2-, 3-, and 4-methylbenzoic acid (Aldrich). Nuclear magnetic resonance analyses were performed on a Bruker WH-400 (400 Hz) instrument.

Synthesis of chemicals. 4-Hydroxy-3-methylbenzoic acid was synthesized from *o*-cresol as described by Komiyama and Hirai (9). This substance was used as a substrate in some cultures and as a standard for GC and GC-MS analyses.

o-Cresol-6_d was synthesized in three steps. To 5.00 g of 2-bromo-6-methylphenol prepared by the method of Pearson et al. (11) in 30 ml of dry dimethyl formamide were added 3.99 g of imidazole and 4.42 g of *t*-butyl dimethylsilyl chloride. The mixture was left overnight; after extraction with hexane-water, 7.94 g of a colorless oil was obtained.

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The compound was over 97% pure as revealed by GC-MS. The ions at *m/z* 243 and 163 corresponded to the loss of $(CH_3)_3C$ and $(CH_3)_3C$-Br, respectively. The nuclear magnetic resonance spectrum of this compound was as follows: 0.22 ppm (6 H, singlet), 1.05 ppm (9 H, singlet), 6.74 ppm (1 H, triplet, J = 8 Hz), 7.06 ppm (1 H, doublet, J = 8 Hz), 7.34 ppm (1 H, doublet, J = 8 Hz). These results confirmed that the compound 2-bromo-6-methyl-phenol-*t*-butyldimethylsilyl ether was obtained, as expected.

Attempts to replace the bromine with deuterium by using metallic potassium or sodium failed. Thus, to 1.0 g of 2-bromo-6-methyl-phenol-*t*-butyldimethylsilyl ether in 12 ml of methanol d_1 was added 50 mg of 10% palladium on carbon and, over a period of 3 h, 342 mg of sodium borodeuteride; 714 mg of compound 2-methyl-6_d-phenol-*t*-butyldimethylsilyl ether was obtained and found to be 95% pure by GC-MS. The ions at *m/z* 223 and 166 corresponded to the molecular ion and to the loss of $(CH_3)_3C$, respectively. An ion peak with one less atomic mass unit and approximately 20% the peak area of the molecular ion indicated that the compound was about 80% deuterium labeled. The nuclear magnetic resonance spectrum of the compound was as follows: 0.22 ppm (6 H, singlet), 1.03 ppm (9 H, singlet), 6.75 ppm (0.2 H, doublet, J = 7 Hz), 6.85 ppm (1 H, triplet, J = 7 Hz), 7.05 ppm (1 H, doublet, J = 7 Hz), 7.12 ppm (1 H, doublet, J = 7 Hz). These results confirmed that the compound 2-methyl-6_d-phenol-*t*-butyldimethylsilyl ether was obtained, as expected.

In the last step, 714 mg of 2-methyl-6_d-phenol-*t*-butyldimethylsilyl ether was added to 10 ml of an ethanol solution containing 1.5 ml of 3 N HCl. The solution was refluxed for 3 h then the residue was purified by flash chromatography on silica gel (eluent, 8% ethyl acetate–hexane) to give 268 mg of 2-methyl-6_d-phenol. After trimethyl silylation with *N,O*-bis(trimethylsilyl)-trifluoroacetamide–acetonitrile, this compound was found to be more than 99% pure by GC-MS. The ions at *m/z* 181 and 166 corresponded to the molecular ion and to the loss of CH_3, respectively (see Fig. 4a). In this case also, the compound was approximately 80% deuterium labeled. The nuclear magnetic resonance spectrum of the compound was as follows: 2.24 ppm (3 H, singlet), 6.72 ppm (0.2 H, doublet, J = 7 Hz), 6.83 ppm (1 H, triplet, J = 7 Hz), 7.07 ppm (1 H, doublet, J = 7 Hz), 7.11 (1 H, doublet, J = 7 Hz). These results confirmed that the expected compound, 2-methyl-6_d-phenol, was obtained. This labeled compound was used as the substrate in some cultures.

RESULTS

Identification of metabolites. Under methanogenic conditions, the anaerobic consortium transformed *o*-cresol into a compound that accumulated in the culture medium. This metabolite was not observed in the *o*-cresol-free control. The accumulated metabolite had the same GC retention time as 3-methylbenzoic acid, which differs from those of isomers substituted in the 2 and 4 positions. The mass spectrum of the derivatized compound exhibited a molecular ion at *m/z* 208, which was consistent with a trimethylsilyl derivatized isomer of methylbenzoic acid (Fig. 1). The peaks at *m/z* 193 and 119 corresponded to the loss of a CH_3 and O-Si$(CH_3)_3$, respectively.

A transient metabolite was also detected in the culture medium. This metabolite was not observed in the *o*-cresol-free control. The mass spectrum of the trimethylsilyl derivatized transient metabolite revealed a molecular ion at *m/z* 296, which was consistent with a derivatized isomer of hydroxy-methylbenzoic acid (Fig. 2). The peaks at *m/z* 281 and 207 corresponded to the loss of CH_3 and O-Si$(CH_3)_3$, respectively. This metabolite also had the same GC retention time as authentic 4-hydroxy-3-methylbenzoic acid.

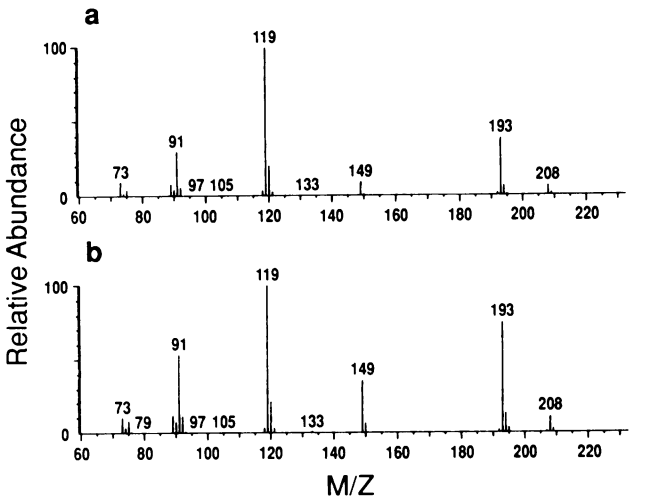

FIG. 1. Mass spectrum of the trimethylsilyl derivatives of the accumulated compound (a) and of authentic 3-methylbenzoic acid (b). Samples were analyzed by GC-MS.

Transformation of *o*-cresol. Proteose peptone must be added to the culture medium for the transformation of *o*-cresol to occur (data not shown). In addition to the starting concentration of proteose peptone in the culture medium, this product must be added after 14 days of incubation of the culture to obtain the transformation of nearly all of the *o*-cresol.

The kinetics of transformation of *o*-cresol by the anaerobic consortium under methanogenic conditions is presented in Fig. 3. After 59 days of incubation, *o*-cresol was nearly completely transformed to 3-methylbenzoic acid, which accumulated in the culture medium. The transient metabolite 4-hydroxy-3-methylbenzoic acid was detected after *o*-cresol metabolism began and reached a maximum concentration

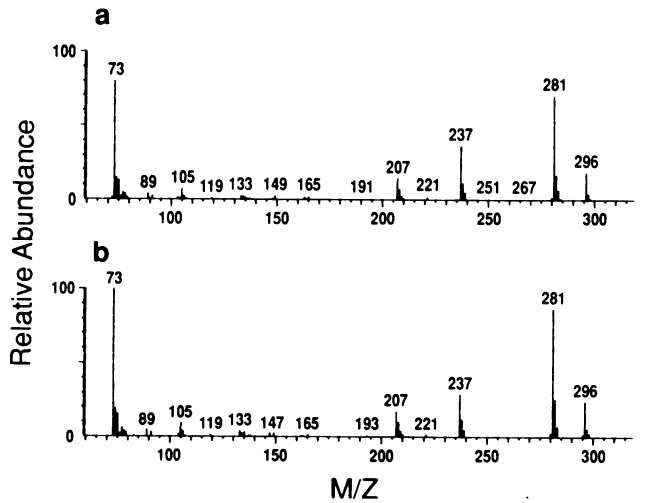

FIG. 2. Mass spectrum of the trimethylsilyl derivatives of the transient metabolite (a) and of authentic 4-hydroxy-3-methylbenzoic acid (b). Samples were analyzed by GC-MS.

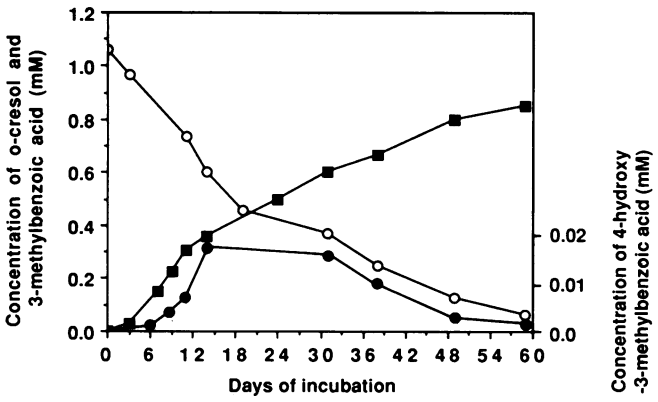

FIG. 3. Kinetics of *o*-cresol transformation under methanogenic conditions. Samples of culture supernatants were analyzed by GC-MS. Symbols: ○, *o*-cresol; ■, 3-methylbenzoic acid; ●, 4-hydroxy-3-methylbenzoic acid.

when about half of the *o*-cresol had been transformed. Thereafter, the concentration of this metabolite in the culture decreased, suggesting that it was an intermediate in *o*-cresol metabolism.

Degradative ability of the consortium. The consortium could not metabolize 3-methylbenzoic acid, as verified by prolonging the incubation of the *o*-cresol culture up to 120 days and also by adding this compound instead of *o*-cresol as a substrate in a culture. Also, potential intermediates of *o*-cresol metabolism such as 2-methylcyclohexanol and 2-hydroxybenzoic acid were not metabolized by the consortium. The addition of 4-hydroxy-3-methylbenzoic acid (150 mg/liter) to cultures instead of *o*-cresol revealed that this compound was transformed to 3-methylbenzoic acid and *o*-cresol (Table 1). The concentration of 4-hydroxy-3-methylbenzoic acid remained constant in sterile controls.

Confirmation of the carboxylated position. Upon transformation of *o*-cresol-6_d by the anaerobic consortium, an accumulating metabolite and a transient metabolite were observed, as shown in Fig. 3 for *o*-cresol. However, the mass spectrum of the accumulated metabolite exhibited a molecular ion at *m/z* 209 (Fig. 4b) instead of *m/z* 208 as obtained previously with *o*-cresol cultures (Fig. 1a). This increase of one atomic mass unit is consistent with a trimethylsilyl derivatized isomer of deuterated methylbenzoic acid. Also, in Fig. 4b the peaks at *m/z* 194 and 120 corresponded to the loss of CH_3 and O-Si $(CH_3)_3$, respectively. Similarly, the mass spectrum of the transient compound revealed a molecular ion at *m/z* 297 (Fig. 4c) instead of *m/z* 296 as previously observed with *o*-cresol cultures (Fig. 2a). This is consistent with a derivatized isomer of deuterated hydroxy-methylbenzoic acid. The peaks at *m/z* 282 and 208 corresponded to the loss of CH_3 and O-Si $(CH_3)_3$, respectively (Fig. 4c). The deuterated metabolites were not detected in sterile control cultures that contained *o*-cresol-6_d.

TABLE 1. Transformation of 4-hydroxy-3-methylbenzoic acid by the anaerobic consortium

Days of incubation	Concn[a] (mM) of:		
	4-Hydroxy-3-methylbenzoic acid	*o*-Cresol	3-Methylbenzoic acid
0	1.00 ± 0.10	<0.10	<0.10
7	0.34 ± 0.14	0.35 ± 0.02	0.16 ± 0.04
14	<0.10	0.28 ± 0.02	0.33 ± 0.03

[a] The GC data presented are averages of at least two analyses.

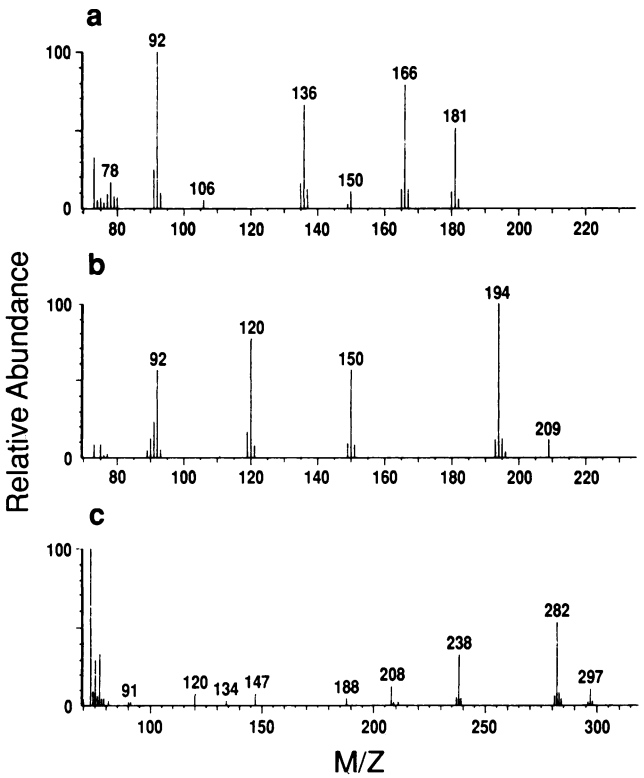

FIG. 4. Mass spectrum of the trimethylsilyl derivatives of *o*-cresol-6_d (a), the accumulated metabolite (b), and the transient metabolite (c). Samples were analyzed by GC-MS.

DISCUSSION

o-Cresol was transformed under methanogenic conditions by our consortium but not degraded to methane as reported by Kaminski et al. (8) with their acclimated consortium. The resulting substance, 3-methylbenzoic acid, was not transformed by our consortium, even though Horowitz et al. (7) have shown that other bacterial consortia can degrade this compound. *o*-Cresol is probably transformed by cometabolism, since proteose peptone was essential for the transformation. Béchard et al. (2) reached a similar conclusion for the degradation of phenol by this consortium, and they suggested that proteose peptone or some degradation products could serve as carbon and energy sources for the growth of the carboxylating bacteria. Phenol (10 days) is transformed more rapidly than *o*-cresol (60 days) by the consortium, and less proteose peptone is needed in the case of phenol.

Based on the metabolites identified in this study, *o*-cresol is transformed by carboxylation and dehydroxylation. These steps are also involved in the transformation of phenol to benzoate by the consortium (2). These reactions were also recently observed with an anaerobic consortium that transformed *m*-cresol (12). However, the metabolite resulting from carboxylation was mainly degraded to methane and was only sporadically dehydroxylated by this consortium. This sporadically produced compound accumulated in the

medium and is an apparent dead-end product, like the carboxylated and dehydroxylated compounds resulting from the transformation of *o*-cresol by our consortium.

The stoichiometric transformation of *o*-cresol into 3-methylbenzoic acid indicated that carboxylation occurred at either the *ortho* or *para* position to the hydroxyl group of *o*-cresol. The identification of the transient metabolite as 4-hydroxy-3-methylbenzoic acid suggests that the consortium introduces a carboxyl function *para* to the phenolic hydroxyl group of *o*-cresol while it is still hydroxylated. The absence of transformation of *p*-cresol by the consortium shown by Béchard et al. (2) is also in agreement with the *para* carboxylation hypothesis. The *para* position of *p*-cresol is blocked by a methyl group, making carboxylation impossible at this position.

o-Cresol-6_d was used to determine whether carboxylation occurred at the *ortho* or *para* position of the hydroxyl group. The deuterium acts as a label to the *ortho* position. If the *ortho* position is carboxylated, then the deuterium label will be lost, producing unlabeled 3-methylbenzoic acid. If the *para* position is carboxylated, then the deuterium label will not be lost and labeled 3-methylbenzoic acid will be produced. The results obtained show that the accumulated methylbenzoic acid and the transient hydroxy-methylbenzoic acid resulting from the transformation of *o*-cresol-6_d are both deuterated, suggesting that the carboxylation occurred in the *para* position and not in the *ortho* position of the hydroxyl group. Sharak Genthner et al. (13) and Zhang et al. (17) have found a similar *para* carboxylation with their consortium with fluorophenols and ^{13}C-1-labeled phenol, respectively. Such a carboxylation followed by a dehydroxylation was also proposed by Roberts et al. (12) with *m*-cresol based on experiments with radioactively labeled compounds.

Sharak Genthner et al. (13) proposed that *p*-hydroxybenzoic acid is probably the intermediate involved in the carboxylation of phenol to benzoate. Zhang et al. (17) suggested that *p*-hydroxybenzoic acid was not a free intermediate in the conversion of phenol to benzoate by their consortium. In our case, the transient metabolite 4-hydroxy-3-methylbenzoic acid is believed to be the intermediate in the conversion of *o*-cresol to 3-methylbenzoic acid. In cultures with 4-hydroxy-3-methylbenzoic acid added as a substrate, dehydroxylation yielded 3-methylbenzoic acid and decarboxylation yielded *o*-cresol. Thus, the carboxylation reaction seems reversible in the presence of a high concentration of 4-hydroxy-3-methylbenzoid acid. However, more data are needed to confirm this hypothesis.

The microorganisms in our consortium are able to carboxylate *o*-cresol even though the consortium was maintained on phenol for a few years. The isolation of the carboxylating microorganisms would be required to determine whether the same microorganisms are implicated in the carboxylation of both substances. The accumulation of 3-methylbenzoate in the culture seems to reflect the inability of the benzoate-degrading microorganisms to metabolize such a substituted benzoate.

The results of our study demonstrated a new pathway for the transformation of *o*-cresol under methanogenic conditions. This pathway has some points in common with the one proposed by Roberts et al. (12) for *m*-cresol. However, it is different from those suggested by Grbić-Galić and Vogel (6), since the aromatic ring is not reduced and the methyl group is not oxidized. *o*-Cresol is carboxylated in the *para* position relative to the phenolic hydroxyl group and is dehydroxylated by the anaerobic consortium to yield 3-methylbenzoic acid.

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